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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 14/705, A61K 38/17, C07K 16/28, G01N 33/53	A2	(11) International Publication Number: WO 99/14241 (43) International Publication Date: 25 March 1999 (25.03.99)																					
(21) International Application Number: PCT/US98/19437 (22) International Filing Date: 17 September 1998 (17.09.98) (30) Priority Data: <table border="0"><tr><td>60/059,119</td><td>17 September 1997 (17.09.97)</td><td>US</td></tr><tr><td>60/059,263</td><td>18 September 1997 (18.09.97)</td><td>US</td></tr><tr><td>60/063,550</td><td>28 October 1997 (28.10.97)</td><td>US</td></tr><tr><td>60/066,770</td><td>12 November 1997 (12.11.97)</td><td>US</td></tr><tr><td>60/066,364</td><td>21 November 1997 (21.11.97)</td><td>US</td></tr><tr><td>60/065,186</td><td>24 November 1997 (24.11.97)</td><td>US</td></tr><tr><td>60/088,026</td><td>4 June 1998 (04.06.98)</td><td>US</td></tr></table> (71) Applicant (for all designated States except US): GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): FONG, Sherman [US/US]; 19 Basinside Way, Alameda, CA 94502 (US). GODDARD, Audrey [CA/US]; 110 Congo Street, San Francisco, CA 94131 (US). GURNEY, Austin, L. [US/US]; 1 Debbie Lane, Belmont, CA 94002 (US). TUMAS, Daniel [US/US]; 3 Rae Avenue, Orinda, CA 94563 (US). WOOD, William, I. [US/US]; 1400 Tarrytown Street, San Mateo, CA 94402 (US).		60/059,119	17 September 1997 (17.09.97)	US	60/059,263	18 September 1997 (18.09.97)	US	60/063,550	28 October 1997 (28.10.97)	US	60/066,770	12 November 1997 (12.11.97)	US	60/066,364	21 November 1997 (21.11.97)	US	60/065,186	24 November 1997 (24.11.97)	US	60/088,026	4 June 1998 (04.06.98)	US	(74) Agents: SCHWARTZ, Timothy, R. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
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(54) Title: COMPOSITIONS AND METHODS FOR THE TREATMENT OF IMMUNE RELATED DISEASES (57) Abstract The present invention relates to a composition containing novel proteins and methods for the diagnosis and treatment of immune related diseases.																							

technology, such as those described hereinabove and in the following textbooks: Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press N.Y., 1989; Ausubel *et al.*, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y., 1989; Innis *et al.*, *PCR Protocols: A Guide to Methods and Applications*, Academic Press, inc., N.Y., 1990; Harlow *et al.*,
 5 *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, 1988; Gait, M.J., *Oligonucleotide Synthesis*, IRL Press, Oxford, 1984; R.I. Freshney, *Animal Cell Culture*, 1987; Coligan *et al.*, *Current Protocols in Immunology*, 1991.

EXAMPLE 1

Isolation of cDNA clones Encoding Human PRO245, PRO217, PRO301, PRO266, PRO335, PRO331 or 10 PRO326

I. Isolation of cDNA Clones Encoding Human PRO245

The extracellular domain (ECD) sequences (including the secretion signal, if any) of from about 950 known secreted proteins from the Swiss-Prot public protein database were used to search expressed sequence tag (EST) databases. The EST databases included public EST databases (e.g., GenBank) and a proprietary
 15 EST DNA database (LIFESEQTM, Incyte Pharmaceuticals, Palo Alto, CA). The search was performed using the computer program BLAST or BLAST2 (Altschul *et al.*, *Methods in Enzymology* 266:460-480 (1996)) as a comparison of the ECD protein sequences to a 6 frame translation of the EST sequence. Those comparisons resulting in a BLAST score of 70 (or in some cases 90) or greater that did not encode known proteins were clustered and assembled into consensus DNA sequences with the program ☐phrap☐ (Phil Green, University
 20 of Washington, Seattle, Washington).

A consensus DNA sequence encoding PRO245 was assembled relative to the other identified EST sequences, wherein the consensus sequence was designated herein as DNA30954 (see Figs. 3A-3B), wherein the polypeptide showed some structural homology to transmembrane protein receptor tyrosine kinase proteins.

25 Based on the DNA30954 consensus sequence, oligonucleotides were synthesized to identify by PCR a cDNA library that contained the sequence of interest and for use as probes to isolate a clone of the full-length coding sequence for PRO245.

A pair of PCR primers (forward and reverse) were synthesized:

forward PCR primer 5'-ATCGTTGTGAAGTTAGTGCCCC-3' (SEQ ID NO:4)

30 reverse PCR primer 5'-ACCTGCGATATCCAACAGAATTG-3' (SEQ ID NO:5)

Additionally, a synthetic oligonucleotide hybridization probe was constructed from the consensus DNA30954 sequence which had the following nucleotide sequence

hybridization probe

5'-GGAAGAGGATACAGTCACTCTGGAAGTATTAGTGCTCCAGCAGTTCC-3' (SEQ ID NO:6)

35 In order to screen several libraries for a source of a full-length clone, DNA from the libraries was screened by PCR amplification with the PCR primer pair identified above. A positive library was then used to isolate clones encoding the PRO245 gene using the probe oligonucleotide and one of the PCR primers.

RNA for construction of the cDNA libraries was isolated from human fetal liver tissue. The cDNA libraries used to isolate the cDNA clones were constructed by standard methods using commercially available
 40 reagents such as those from Invitrogen, San Diego, CA. The cDNA was primed with oligo dT containing a NotI site, linked with blunt to SalI hemikinased adaptors, cleaved with NotI, sized appropriately by gel electrophoresis, and cloned in a defined orientation into a suitable cloning vector (such as pRKB or pRKD;

pRK5B is a precursor of pRK5D that does not contain the SfiI site; see, Holmes et al., *Science*, 253:1278-1280 (1991)) in the unique XhoI and NotI sites.

DNA sequencing of the clones isolated as described above gave the full-length DNA sequence for PRO245 [herein designated as UNQ219 (DNA35638)] and the derived protein sequence for PRO245.

5 The entire nucleotide sequence of UNQ219 (DNA35638) is shown in Figure 1 (SEQ ID NO:1). Clone UNQ219 (DNA35638) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 89-91 [Kozak et al., *supra*] and ending at the stop codon at nucleotide positions 1025-1027 (Fig. 1; SEQ ID NO:1). The predicted polypeptide precursor is 312 amino acids long (Fig. 2). Clone UNQ219 (DNA35638) has been deposited with ATCC on September 17, 1997 and is assigned ATCC
10 Deposit No. 209265.

Analysis of the amino acid sequence of the full-length PRO245 suggests that a portion of it possesses 60% amino acid identity with the human c-myc protein and, therefore, may be a new member of the transmembrane protein receptor tyrosine kinase family.

II. Isolation of cDNA clones Encoding PRO217

15 The extracellular domain (ECD) sequences (including the secretion signal sequence, if any) from about 950 known secreted proteins from the Swiss-Prot public database were used to search EST databases. The EST databases included public databases (e.g., Dayhof, GenBank), and proprietary databases (e.g., LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA). The search was performed using the computer program BLAST or BLAST2 (Altschul, SF and Gish (1996), *Methods in Enzymology* 266: 460-80 (1996);
20 <http://blast.wustl.edu/blast/README.html>) as a comparison of the ECD protein sequences to a 6 frame translation of the EST sequences. Those comparisons with a Blast score of 70 (or in some cases 90) or greater that did not encode known proteins were clustered and assembled into consensus DNA sequences with the program "phrap" (Phil Green, University of Washington, Seattle, WA; (<http://bozeman.mbt.washington.edu/phrap.docs/phrap.html>)).

25 Consensus DNA sequences encoding EGF-like homologues were assembled (DNA28726, SEQ ID NO: 19, Fig. 7A; DNA28730, SEQ ID NO: 21, Fig. 7B and DNA28760, SEQ ID NO: 20, Fig. 7C) using phrap. In some cases, the consensus DNA sequence was extended using repeated cycles of blast and phrap to extend the consensus sequence as far as possible using the three sources of EST sequences listed above. (Indicated as second alignment figure).

30 Based on this consensus sequence, oligonucleotides were synthesized: 1) to identify by PCR a cDNA library that contained the sequence of interest, and 2) for use as probes to isolate a clone of the full-length coding sequence. The pair of forward and reverse PCR primers (notated as *.f and *.r, respectively) may range from 20 to 30 nucleotides (typically 24), and are designed to give a PCR product of 100-1000 bp in length. The probe sequences (notated as *.p) are typically 40-55 bp (typically 50) in length. In some cases additional
35 oligonucleotides are synthesized when the consensus sequence is greater than 1-1.5 kbp. In order to screen several libraries for a source of a full-length clone, DNA from the libraries was screened by PCR amplification, as per Ausubel et al., *Current Protocols in Molecular Biology*, with the PCR primer pair. A positive library was then used to isolate clones encoding the gene of interest by the *in vivo* cloning procedure using the probe oligonucleotide and one of the PCR primers. This library was used to isolate DNA32279, DNA32292 and
40 DNA33094 was fetal kidney, fetal lung and fetal lung, respectively.

RNA for the construction of the cDNA libraries was isolated using standard isolation protocols, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, from tissue or cell line sources or it was purchased from

order to screen several libraries for a full-length clone, DNA from the libraries was screened by PCR amplification, as per Ausubel et al., Current Protocols in Molecular Biology, with the PCR primer pair. A positive library was then used to isolate clones encoding the gene of interest by the *in vivo* cloning procedure using the probe oligonucleotide and one of the primer pairs.

- 5 A number of PCR primers (forward and reverse) were synthesized as shown in Figure 30C and Figure 31 (forward SEQ ID NOS:271-274; reverse SEQ ID NOS:275-277) and yet another primer, SEQ ID NO:278 shown in Figure 31 for determination of PRO335. For determination of PRO40981, the primers are shown in Figure 36, (forward is SEQ ID NO:295; reverse is SEQ ID NO:296; and the other is SEQ ID NO:297). For the determination of PRO326, a 5' splice variant of PRO335, the primers used are shown in
10 Figures 40 and Figures 41.

In order to screen several libraries for a source of a full-length clone, DNA from the libraries was screened by PCR amplification with the PCR primer pair identified above. A positive library was then used to isolate clones encoding the PRO335, PRO331 or PRO326 gene using the probe oligonucleotide and one of the PCR primers.

- 15 RNA for construction of the cDNA libraries was isolated from human fetal kidney tissue (PRO335 and PRO326) and human fetal brain (PRO331). The cDNA libraries used to isolate the cDNA clones were constructed by standard methods using commercially available reagents such as those from Invitrogen, San Diego, CA. The cDNA was primed with oligo dT containing a NotI site, linked with blunt to SalI hemikinased adaptors, cleaved with NotI, sized appropriately by gel electrophoresis, and cloned in a defined
20 orientation into a suitable cloning vector (such as pRKB or pRKD; pRK5B is a precursor of pRK5D that does not contain the SfiI site; see, Holmes et al., Science, 253:1278-1280 (1991)) in the unique XhoI and NotI sites.

- DNA sequencing of the clones isolated as described above gave the full-length DNA sequence for PRO335, PRO331 or PRO326 [herein designated as SEQ ID NOS:261, 279 or 298, and the derived protein
25 sequence for PRO335, PRO331 or PRO326.

The entire nucleotide sequences are shown in Figures 25A-B, 32 and 37A-C. The nucleic acid shown in Figure 32 has been deposited with the ATCC on 7 November 1997 and is assigned ATCC Accession No. 209439.

- Analysis of the amino acid sequence of the full-length PRO335, PRO331 or PRO326 polypeptide
30 suggests that portions of it possess significant homology to the LIG-1 protein as shown in Figures 28A-28C, 34A-34E and 39A-39D, thereby indicating that PRO335, PRO331 and PRO326 may be a novel LIG-1-related protein.

EXAMPLE 2

Stimulatory Activity in Mixed Lymphocyte Reaction (MLR) Assay

- 35 This example shows that the polypeptides of the invention are active as a stimulator of the proliferation of stimulated T-lymphocytes. Compounds which stimulate proliferation of lymphocytes are useful therapeutically where enhancement of an immune response is beneficial. Compounds which inhibit proliferation of lymphocytes are useful therapeutically where suppression of an immune response is beneficial. A therapeutic agent may take the form of antagonists of the polypeptide of the invention, for
40 example, murine-human chimeric, humanized or human antibodies against the polypeptide.

The basic protocol for this assay is described in Current Protocols in Immunology, unit 3.12; edited by J E Coligan, A M Kruisbeek, D H Marglies, E M Shevach, W Strober, National Institutes of Health, Published by John Wiley & Sons, Inc.

More specifically, in one assay variant, peripheral blood mononuclear cells (PBMC) are isolated from mammalian individuals, for example a human volunteer, by leukopheresis (one donor will supply stimulator PBMCs, the other donor will supply responder PBMCs). If desired, the cells are frozen in fetal bovine serum and DMSO after isolation. Frozen cells may be thawed overnight in assay media (37 °C, 5% CO₂) and then washed and resuspended to 3 x 10⁶ cells/ml of assay media (RPMI; 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, 1% HEPES, 1% non-essential amino acids, 1% pyruvate).

The stimulator PBMCs are prepared by irradiating the cells (about 3000 Rads). The assay is prepared by plating in triplicate wells a mixture of:

100 µl of test sample diluted to 1% or to 0.1%

50 µl of irradiated stimulator cells and

50 µl of responder PBMC cells.

100 microliters of cell culture media or 100 microliter of CD4-IgG is used as the control. The wells are then incubated at 37 °C, 5% CO₂ for 4 days. On day 5 each well is pulsed with tritiated thymidine (1.0 mCi/well; Amersham). After 6 hours the cells are washed 3 times and then the uptake of the label is evaluated.

In another variant of this assay, PBMCs are isolated from the spleens of Balb/c mice and C57B6 mice. The cells are teased from freshly harvested spleens in assay media (RPMI; 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, 1% HEPES, 1% non-essential amino acids, 1% pyruvate) and the PBMCs are isolated by overlaying these cells over Lympholyte M (Organon Teknika), centrifuging at 2000 rpm for 20 minutes, collecting and washing the mononuclear cell layer in assay media and resuspending the cells to 1 x 10⁷ cells/ml of assay media. The assay is then conducted as described above. The results of this assay for compounds of the invention are shown below. Positive increases over control are considered positive with increases of greater than or equal to 180% being preferred. However, any value greater than control indicates a stimulatory effect for the test protein.

Table

	<u>PRO</u>	<u>PRO Concentration</u>	<u>Percent Increase Over Control</u>
30	PRO245	0.1%	189.7
	"	0.1%	193.7
	"	1.0%	212.5
	"	1.0%	300.5
	PRO217	0.1%	74.5
35	"	1.0%	89.5
	"	0.99 nM	97.0
	"	9.9 nM	122.3
	"	0.25 nM	144.8
	"	2.5 nM	126.9
40	PRO301	50.0%	109.4
	"	70.0 nM	133.7

	"	700.0 nM	83.6
		0.1%	58.7
	PRO301 (cont.)	1.0%	127.7
	"	0.1%	181.7
5	"	1.0%	187.3
	"	0.1%	127.5
	"	1.0%	108.3
	PRO266	0.1%	136.4
	"	0.1%	139.2
10	"	1.0%	189.8
	"	1.0%	245.1
	PRO335	50.0%	91.0
	"	50.0%	103.8
	"	0.1%	130.0
15	"	1.0%	180.2
	PRO331	50.0%	155.5
	"	0.1%	169.3
	"	1.0%	128.1
	"	0.1%	129.3
20	"	1.0%	162.5
	PRO326	50.0%	91.0
	"	50.0%	103.8
	"	0.1%	130.0
	"	1.0%	180.2

25

EXAMPLE 3**Skin Vascular Permeability Assay**

This assay shows that certain polypeptides of the invention stimulate an immune response and induce inflammation by inducing mononuclear cell, eosinophil and PMN infiltration at the site of injection of the animal. This skin vascular permeability assay is conducted as follows. Hairless guinea pigs weighing 350 grams or more are anesthetized with ketamine (75-80 mg/Kg) and 5 mg/Kg xylazine intramuscularly (IM). A sample of purified polypeptide of the invention or a conditioned media test sample is injected intradermally onto the backs of the test animals with 100 uL per injection site. It is possible to have about 10-30, preferably about 16-24, injection sites per animal. One mL of Evans blue dye (1% in physiologic buffered saline) is injected intracardially. Blemishes at the injection sites are then measured (mm diameter) at 1hr and 6 hr post injection. Animals were sacrificed at 6 hrs after injection. Each skin injection site was biopsied and fixed in formalin. The skins were then prepared for histopathologic evaluation. Each site was evaluated for inflammatory cell infiltration into the skin. Sites with visible inflammatory cell inflammation were scored as positive. Inflammatory cells can be neutrophilic, eosinophilic, monocytic or lymphocytic. The results of this test for compounds of the invention is shown below.

In the Table below, at least a minimal perivascular infiltrate at the injection site is scored as positive, no infiltrate at the site of injection is scored as negative.

Table

<u>PRO</u>	<u>Hours Post Injection</u>	<u>Infiltrate Designation</u>
PRO245	24 hr	positive
PRO217	24 hr	positive
5 PRO301	24 hr	positive
PRO266	24 hr	positive
PRO335	24 hr	positive
PRO331	24 hr	positive
PRO326	24 hr	positive

10

EXAMPLE 4

Human Co-Stimulation Assay

In addition to the activation signal mediated by the T cell receptor, T cell activation requires a costimulatory signal. One costimulatory signal is generated by the interaction of B7 (CD3) with CD28. In this assay, 96 well plates are coated with CD3 with or without CD28 and then human peripheral blood lymphocytes followed by a test protein, are added. Proliferation of the lymphocytes is determined by tritiated thymidine uptake. A positive assay indicates that the test protein provided a stimulatory signal for lymphocyte proliferation.

Material:

- 1) Hyclone D-PBS without Calcium, Magnesium
- 20 2) Nunc 96 well certified plates #4-39454
- 3) Anti-human CD3 Amac 0178 200 µg/ml stock
- 4) Anti-human CD28 Biodesign P42235M
- 5) Media: Gibco RPMI 1640 + 10 % Intergen #1020-90 FBS, 1% Glu, 1% P/S, 50 µg/ml Gentamycin, 10 mM Hepes. Fresh for each assay.
- 25 6) Tritiated Thymidine
- 7) Frozen human peripheral blood lymphocytes (PBL) collected via a leukaphoresis procedure

Plates are prepared by coating 96 well flat bottom plates with anti-CD3 antibody (Amac) or anti-CD28 antibody (Biodesign) or both in Hyclone D-PBS without calcium and magnesium. Anti-CD3 antibody is used at 10 ng/well (50 µl of 200 ng/ml) and anti-CD28 antibody at 25 ng/well (50 µl of 0.5 µg/ml) in 100 µl total volume.

PBLs are isolated from human donors using standard leukaphoresis methods. The cell preparations are frozen in 50% fetal bovine serum and 50% DMSO until the assay is conducted. Cells are prepared by thawing and washing PBLs in media, resuspending PBLs in 25 mls of media and incubating at 37°C, 5% CO₂ overnight.

35 In the assay procedure, the coated plate is washed twice with PBS and the PBLs are washed and resuspended to 1×10^6 cells/ml using 100 µL /well. 100 ul of a test protein or control media are added to the plate making a total volume per well of 200 µL. The plate is incubated for 72 hours. The plate is then pulsed for 6 hours with tritiated thymidine (1 mCi/well; Amersham) and the PBLs are harvested from the plates and evaluated for uptake of the tritiated thymidine.

EXAMPLE 5

In situ Hybridization

In situ hybridization is a powerful and versatile technique for the detection and localization of nucleic acid sequences within cell or tissue preparations. It may be useful, for example, to identify sites of gene expression, analyze the tissue distribution of transcription, identify and localize viral infection, follow changes in specific mRNA synthesis and aid in chromosome mapping.

In situ hybridization was performed following an optimized version of the protocol by Lu and Gillett, Cell Vision 1: 169-176 (1994), using PCR-generated ³³P-labeled riboprobes. Briefly, formalin-fixed, paraffin-embedded human tissues were sectioned, deparaffinized, deproteinized in proteinase K (20 g/ml) for 15 minutes at 37°C, and further processed for *in situ* hybridization as described by Lu and Gillett, *supra*. A [³³P] UTP-labeled antisense riboprobe was generated from a PCR product and hybridized at 55°C overnight. The slides were dipped in Kodak NTB2 nuclear track emulsion and exposed for 4 weeks.

³³P-Riboprobe synthesis

- 6.0 µl (125 mCi) of ³³P-UTP (Amersham BF 1002, SA<2000 Ci/mmol) were speed vac dried.
- To each tube containing dried ³³P-UTP, the following ingredients were added:
- 2.0 µl 5x transcription buffer
 - 1.0 µl DTT (100 mM)
 - 2.0 µl NTP mix (2.5 mM : 10 µl; each of 10 mM GTP, CTP & ATP + 10 µl H₂O)
 - 1.0 µl UTP (50 µM)
 - 1.0 µl Rnasin
 - 1.0 µl DNA template (1 µg)
 - 1.0 µl H₂O

The tubes were incubated at 37°C for one hour. 1.0 µL RQ1 DNase were added, followed by incubation at 37°C for 15 minutes. 90 µL TE (10 mM Tris pH 7.6/1mM EDTA pH 8.0) were added, and the mixture was pipetted onto DE81 paper. The remaining solution was loaded in a Microcon-50 ultrafiltration unit, and spun using program 10 (6 minutes). The filtration unit was inverted over a second tube and spun using program 2 (3 minutes). After the final recovery spin, 100 µL TE were added. 1 µL of the final product was pipetted on DE81 paper and counted in 6 ml of Biofluor II.

The probe was run on a TBE/urea gel. 1-3 µL of the probe or 5 µL of RNA Mrk III were added to 30 µL of loading buffer. After heating on a 95°C heat block for three minutes, the gel was immediately placed on ice. The wells of gel were flushed, the sample loaded, and run at 180-250 volts for 45 minutes. The gel was wrapped in saran wrap and exposed to XAR film with an intensifying screen in -70°C freezer one hour to overnight.

³³P-Hybridization

Pretreatment of frozen sections The slides were removed from the freezer, placed on aluminium trays and thawed at room temperature for 5 minutes. The trays were placed in 55°C incubator for five minutes to reduce condensation. The slides were fixed for 10 minutes in 4% paraformaldehyde on ice in the fume hood, and washed in 0.5 x SSC for 5 minutes, at room temperature (25 ml 20 x SSC + 975 ml SQ H₂O). After deproteinization in 0.5 µg/ml proteinase K for 10 minutes at 37°C (12.5 µL of 10 mg/ml stock in 250 ml

prewarmed RNase-free RNase buffer), the sections were washed in 0.5 x SSC for 10 minutes at room temperature. The sections were dehydrated in 70%, 95%, 100% ethanol, 2 minutes each.

Pretreatment of paraffin-embedded sections The slides were deparaffinized, placed in SQ H₂O, and rinsed twice in 2 x SSC at room temperature, for 5 minutes each time. The sections were deproteinized in 20 µg/ml proteinase K (500 µL of 10 mg/ml in 250 ml RNase-free RNase buffer; 37°C, 15 minutes) - human embryo, or 8 x proteinase K (100 µL in 250 ml RNase buffer, 37°C, 30 minutes) - formalin tissues. Subsequent rinsing in 0.5 x SSC and dehydration were performed as described above.

Prehybridization The slides were laid out in plastic box lined with Box buffer (4 x SSC, 50% formamide) - saturated filter paper. The tissue was covered with 50 µL of hybridization buffer (3.75g Dextran Sulfate + 6 ml SQ H₂O), vortexed and heated in the microwave for 2 minutes with the cap loosened. After cooling on ice, 18.75 ml formamide, 3.75 ml 20 x SSC and 9 ml SQ H₂O were added, the tissue was vortexed well, and incubated at 42°C for 1-4 hours.

Hybridization 1.0 x 10⁶ cpm probe and 1.0 µL tRNA (50 mg/ml stock) per slide were heated at 95°C for 3 minutes. The slides were cooled on ice, and 48 µL hybridization buffer were added per slide. After vortexing, 50 µL ³³P mix were added to 50 µL prehybridization on slide. The slides were incubated overnight at 55°C.

Washes Washing was done 2x10 minutes with 2xSSC, EDTA at room temperature (400 ml 20 x SSC + 16 ml 0.25M EDTA, V_f=4L), followed by RNaseA treatment at 37°C for 30 minutes (500 µL of 10 mg/ml in 250 ml RNase buffer = 20 µg/ml). The slides were washed 2x10 minutes with 2 x SSC, EDTA at room temperature. The stringency wash conditions were as follows: 2 hours at 55°C, 0.1 x SSC, EDTA (20 ml 20 x SSC + 16 ml EDTA, V_f=4L).

DNA 35638 (1 TM receptor)

Expression was observed in the endothelium lining of a subset of fetal and placental vessels.

Endothelial

expression was confined to these tissue blocks. Expression was also observed over intermediate trophoblast cells of placenta.

Oligo C-120N: (SEQ ID NO:311)

GGA TTC TAA TAC GAC TCA CTA TAG GGC TGC GGC GGC TCA GGT CTT CAG TT

30

Oligo c-120P (SEQ ID NO:312)

CTA TGA AAT TAA CCC TCA CTA AAG GGA GCA TGG GAT GGG GAG GGA TAC GG

DNA 33094 (EGF Homolog)

A highly distinctive expression pattern was observed. In the human embryo expression was observed in outer smooth muscle layer of the GI tract, respiratory cartilage, branching respiratory epithelium, osteoblasts, tendons, gonad, in the optic nerve head and developing dermis. In the adult, expression was observed in the epidermal pegs of the chimp tongue, the basal epithelial / myoepithelial cells of the prostate and urinary bladder. Expression was also found in the alveolar lining cells of the adult lung, mesenchymal cells juxtaposed to erectile tissue in the penis and the cerebral cortex (probably glial cells). In the kidney, expression was only seen in disease, in cells surrounding thyroidized renal tubules.

40